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## ALTERATION OF RESTRICTION ENDONUCLEASE SPECIFICITY BY GENETIC SELECTION

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### BACKGROUND OF THE INVENTION

Type II restriction endonucleases are a class of enzymes that occur naturally in bacteria where they serve as host defense systems, functioning to prevent infection by foreign DNA molecules such as bacteriophage and plasmids that would otherwise destroy or parasitize them. In this defense system, foreign DNA is restricted (cleaved) while host DNA is protected due to modification of the recognition sites by a cognate DNA methyltransferase. This relationship with a DNA methylase ensures that the endonuclease maintains an extremely high degree of specificity. In the course of evolution, any variants with non-cognate restriction activity are subject to strong selection pressure in the form of DNA damage encountered by the bacterial cell.

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The remarkable substrate specificity of restriction endonucleases has contributed greatly to the biotechnology revolution. Purification of restriction endonucleases from bacteria allows these enzymes to be used in numerous laboratory applications from gene cloning to mutation detection.

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Type II restriction endonucleases typically recognize a DNA sequence of 4-8 base pairs (bp). Of the greater than 3000 enzymes characterized so far, 228 distinct substrate specificities have been identified. (Roberts and Macelis, *Nucl. Acids Res.* 29:268-269 (2001)). The substrate specificity of a restriction endonuclease usually involves single site recognition (e.g. 5'-

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AGATCT-3') However, a relatively common feature is recognition of a degenerate sequence. For example, *Bst*YI recognizes 5'-RGATCY-3' (where R = A or G and Y = C or T). Recognition of a degenerate sequence often limits the utility of a restriction enzyme in laboratory applications since cleavage frequency is excessive. Statistically, an enzyme recognizing a 6-bp sequence cleaves every 4096 bp while an enzyme recognizing 5'-RGATCY-3' cleaves every 1024 bp on average in a non-biased genome. Restriction endonucleases that cut infrequently (e.g. 8-bp cutting enzymes that cleave every 65,536) are rarely found in nature. Therefore, many engineering efforts are focused on creating less frequent cutters out of existing 6-bp cutters.

More than ten years of endonuclease engineering has resulted in only limited success in altering the substrate specificity of an existing restriction endonuclease. One important example is an attempt to engineer an 8-bp cutting enzyme from *EcoRV* (5'-GATATC-3') by using rational protein design based on the high resolution structures of *EcoRV* complexed with alternate 8-bp substrates (Horton and Perona, *J. Biol. Chem.* 273:21721-21729 (1998)). In this case, rational protein design pertained to creating one or more specific amino acid substitutions by site-directed mutagenesis of the cloned gene fragment. A conclusion of this effort was that the determinants of altering substrate specificity are difficult to predict even after crystallographic analysis of an endonuclease/DNA substrate complex (Lanio, et al., *Protein Eng.* 13:275-281 (2000)). The most promising *EcoRV* variant was derived from a semi-rational approach where twenty-two amino acid residues were chosen

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for randomization based on examination of the three-dimensional structure. Clones of interest were selected by *in vitro* analysis of cleavage activity and specificity. From this effort, a triple mutant was identified which exhibited a 25-fold higher rate of cleaving *EcoRV* sites flanked by AT rather than GC base pairs. (Lanio, et al., *J. Mol. Biol.* 283:59-69 (1998)).

Many other studies have been conducted to investigate and possibly alter the substrate specificity of the restriction enzymes *BamHI* (Dorner and Schildkraut, *Nucl. Acids Res.* 22:1068-1074 (1994), Dorner, et al., *J. Mol. Biol.* 285:1515-1523 (1999), Whitaker, et al., *J. Mol. Biol.* 285:1525-1536 (1999), Newman, et al., *Science* 269:656-663 (1995), Newman, et al., *Nature* 368:660-664 (1994)) and *EcoRI* (Ivanenko, et al., *J. Biol. Chem.* 379:459-465 (1998), Heitman and Model, *Proteins* 7:185-197 (1990), Heitman, *Bioessays* 14:445-454 (1992), Muir, et al., *J. Mol. Biol.* 274:722-737 (1997), Flores, et al., *Gene* 157:295-301 (1995)). Again, structure-based rational or semi-rational design approaches were employed with no absolute change of specificity reported. To date, a total of twelve structures of restriction enzymes have been determined (Pingoud and Jeltsch, *Nucl. Acids Res.* 29:3705-3727 (2001)). From these structures, it is becoming clear that substrate recognition does not adhere to a distinct set of rules. Consequently, the likelihood of engineering novel substrate specificities into existing endonucleases by purely rational design methods remains low. Furthermore, protein structure determination remains to be a costly and time-consuming endeavor.

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Consequently, a rapid and more successful method of endonuclease engineering is required to identify amino acid substitutions responsible for altering substrate specificity without the requirement of protein structural information.

### **SUMMARY OF THE INVENTION**

10       The present invention provides a novel method for altering the DNA recognition and cleavage specificity of an endonuclease. Prior knowledge of the endonuclease three-dimensional structure and/or knowledge of the amino acids responsible for substrate recognition are not required.

15       In an embodiment of the invention, a method is provided for altering an endonuclease recognition site specificity, that includes: subjecting a mutagenized endonuclease gene library to a genetic selection in a population of prokaryotic host cells expressing one or more non-cognate DNA methyltransferases, wherein the genetic selection selects for viable cells in the population; and identifying whether the viable cells express an active mutated endonuclease with an altered recognition site specificity.

20       The mutagenized endonuclease gene library may be formed by: error prone PCR, chemical mutagenesis, assembly PCR, DNA shuffling, *in vivo* mutagenesis, cassette mutagenesis, recursive-ensemble mutagenesis or exponential ensemble mutagenesis.

The endonuclease activity may be attenuated by modifying the mutagenized endonuclease gene library using modification means selected from: creating an amber codon within the open reading frame; creating an opal codon within the open reading frame; changing the start codon to GTG or TTG; mutating the RBS sequence or utilizing a T7 expression vector wherein the host cell is T7 RNA polymerase negative.

In addition to the above, viable prokaryotic host cells may be pooled and plasmid DNA isolated from the cells where the plasmid DNA encodes mutagenized endonuclease genes from the library and transforming the plasmid DNA into a population of indicator cells for detecting DNA damage. The mutagenized endonuclease genes may be subjected to repeated genetic selections in the population of host cells described above and in the population of indicator cells where the genetic selection in the population of indicator cells includes a first population of indicator cells lacking non-cognate methylase and a second population of indicator cells expressing the non-cognate methylases.

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The sequence of the altered recognition site can be determined where the altered specificity for the site may be relaxed recognition-site specificity, increased recognition-site specificity or alternate recognition-site specificity.

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In a further embodiment of the invention, a method is provided for altering recognition site specificity of an endonuclease, that includes: creating a mutagenized endonuclease gene expression plasmid library from a target endonuclease gene and

transforming prokaryotic cells with the mutagenized library, wherein  
the prokaryotic cells express one or more non-cognate  
methyltransferase; selecting prokaryotic cells which are viable after  
transformation and isolating plasmid DNA from the viable cells;  
5 determining whether the isolated plasmid DNA encodes an active  
endonuclease by transforming the plasmid DNA into DNA damage  
indicator cells; screening the plasmid DNA encoding the active  
endonuclease for altered specificity; and optionally repeating the  
above protocol to obtain the endonuclease with altered recognition-  
site specificity. An example of the above method is an endonuclease  
10 having an altered recognition site derived from BstYI or NotI.

In a further embodiment of the invention, a method is  
provided for modifying recognition-site specificity of an  
15 endonuclease from a parent specificity to a target specificity, that  
include obtaining a sequence for a plurality of mutated  
endonucleases obtained by the methods described above to  
determine the mutation for each mutated endonuclease; and  
mutating a gene encoding the endonuclease to produce one or  
20 more of the mutations identified above so as to provide the target  
specificity for the endonuclease.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

25 Figure 1A is an outline of the genetic selection procedure.  
Rgene\*, mutated endonuclease gene library; Mgene, non-  
cognate DNA methylase gene.

Figure 1B is an outline of the genetic selection procedure as applied to isolate variants of *Bst*YI with AGATCT specificity.

5 Figure 2 is the nucleotide (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) of *Bst*YI endonuclease.

10 Figure 3 is the DNA cleavage pattern of clone NN1 vs. wild-type *Bst*YI assayed on 0.5 µg substrate pUCAdenoXba. Lanes 1-4 represent cleavage with 0.5, 1.0, 2.0 and 4.0 units wild-type *Bst*YI for 1 hour, respectively. Lanes 5-9 represent cleavage with an excess of clone NN1 for 30, 60, 90 and 120 min, respectively. Lane 9 is complete digestion of 0.5 µg pUCAdenoXba with *Bg*II to show the pattern of restriction at 5'-AGATCT-3' only. All reactions were incubated at 55°C.

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Figure 4 is the DNA cleavage characteristics of clone NN1 vs. wild-type *Bst*YI assayed on radiolabeled DNA substrates (60 bp) each containing one of the three unique *Bst*YI recognition sites.

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Figure 4A is cleavage of the 5'-AGATCT-3' site. Figure 4B is cleavage of the 5'-GGATCC site. Figure 4C is cleavage of the 5'-AGATCC-3' site (bottom strand is 5'-GGATCT-3'). In each part, lane 1 is the substrate only. Lanes 2-5 represent cleavage with wild-type *Bst*YI for 2, 4, 6, and 10 min, respectively. Lanes 6-9 represent cleavage with clone NN1 for 5, 10, 20, and 30 min, respectively. All reactions were incubated at 60°C.

**DETAILED DESCRIPTION OF THE INVENTION****Definitions**

5                  For convenience, certain terms employed in the specification, examples and appended claims are collected here.

10                "Non-cognate methylase" refers herein to a DNA methyltransferase possessing a substrate specificity that does not protect a target recognition site(s) of a given endonuclease.

15                "Mutation" refers herein to any gene alteration including DNA rearrangement; nucleotide(s) substitution, addition and/or deletion that results in expression of an endonuclease possessing an altered amino acid sequence.

20                "Active" refers herein to an endonuclease capable of binding a recognition site(s) or capable of binding and cleaving a recognition site(s). Binding activity may be determined, for example, by an electrophoretic mobility shift assay (Thompson and Landy, *Nucl. Acids Res.* 16:9687-9705 (1988)), filter binding assay (Zhenyu Zhu, New England Biolabs) or may be reported by an indicator strain capable of sensing site-specific DNA binding. Cleavage activity may be determined *in vivo* by an indicator strain capable of reporting DNA damage or *in vitro* by incubating cell extract or partially purified endonuclease with an appropriate DNA substrate (Xu and Schildkraut, *J.Biol. Chem.* 266: 4425-4429 (1991)).

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“Recognition site” refers herein to an uninterrupted or interrupted DNA sequence to which an endonuclease is preferentially bound. DNA cleavage by the endonuclease may occur within or outside of the recognition sequence.

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“Tolerated” refers herein to maintenance of cell viability through DNA methylation protection.

10 “Attenuation” refers herein to intentionally decreasing the *in vivo* DNA damaging effects of a given endonuclease gene or gene library. Attenuation may occur at the level of transcription, translation, or by alteration of the endonuclease specific activity by mutagenesis.

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“Altered specificity” refers herein to any measurable endonuclease activity which differs from the wild-type or parent endonuclease. Altered specificity includes relaxed specificity, increased specificity or alternate specificity.

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“Relaxed specificity” refers herein to increased promiscuity of an endonuclease with respect to its recognition site.

5 "Endonuclease gene library" refers herein to a collection of genes where a majority of the library members are unique with respect to their nucleotide sequence (where a single nucleotide difference between two members qualifies as being unique). The library may be derived from one or more endonuclease gene or each member may be artificially constructed to possess the general characteristics of an endonuclease gene sequence.

10 In an embodiment of the invention, we provide a method for the genetic selection of endonuclease variants possessing altered substrate specificity as compared to the parent endonuclease. The parent endonuclease may be the product of a wild-type endonuclease gene isolated from nature or the parent endonuclease may itself be a variant isolated by any other means. For example, in the development of an endonuclease 15 possessing an altered recognition site specificity, an important first step may be to isolate a variant with a relaxed specificity. Once the new recognition sites are determined, one or more non-cognate DNA methylases can be employed *in vivo* to protect 20 the host genomic DNA and thus serve as an enabling factor in the genetic selection procedure of the present invention. The selection of a relaxed specificity variant may be accomplished by transforming a mutagenized endonuclease gene library into a DNA-damage indicator strain protected by a cognate DNA 25 methylase (see example 2 and Heitman and Model, *EMBO J.* 9:3369-3378 (1990)).

In an embodiment of the invention, we subject a mutated endonuclease gene library to a plurality of genetic selections in

5           *E. coli* or other suitable prokaryotic host. While the method may  
be conducted with a minimum of two genetic selections, three or  
more selections are preferred to reduce false positives. The  
procedure yields active variants possessing a high likelihood of  
10 having one or more mutations which are either necessary or  
informative for altering the substrate specificity of the  
endonuclease. The desired substrate specificity is determined by  
protecting the host DNA with one or more non-cognate DNA  
methyltransferase. The host DNA may be methylated at an  
15 alternate recognition site(s) to isolate variants with an alternate  
cleavage preference or the host DNA may be methylated at one  
or more sub-sites to isolate variants with cleavage preference  
towards one or more sub-sites.

15           During the *in vivo* selection process, host bacterial cells  
carrying endonuclease variants with DNA cleavage activity  
outside the spectrum of host DNA methylation are eliminated or  
at least are strongly selected against due to reduced growth.  
Therefore, this approach involving laboratory genetic selection  
20 steps imposed upon a randomly mutated endonuclease gene  
may be considered to be analogous to the evolutionary selection  
process dependent upon native host protection by a cognate  
DNA methyltransferase.

25           A protocol we have developed to achieve *in vivo* selection  
process (see Figure 1) includes one or more of the following  
steps:

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(i) generating a mutated endonuclease library within an expression vector or plasmid.

5 (ii) introducing the endonuclease library into host cells pre-modified with a non-cognate pattern of methylation. The transformed cells are plated on media containing an inducer molecule to maximize elimination of host cells harboring endonuclease variants with DNA cleavage activity outside the spectrum of host DNA methylation.

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(iii) pooling survivors and plasmid DNA from the cells. Clones expressing active variants are isolated by transforming the endonuclease plasmids only into an indicator strain for DNA damage, preferably ER1992 (U.S. Patent No. 5,498,535). The 15 transformed cells are plated on media void of the inducer molecule to avoid lethal levels of DNA damage, as the indicator strain is not protected by methylation (or only partially protected) in this step. The media does contain the substrate X-gal to allow individual colonies to report significant levels of cellular DNA damage upon induction of the SOS repair response 20 and expression of a *dinD1::lacZ* gene fusion.

25 (iv) active endonuclease clones may be isolated by culturing individual dark blue colonies for a short time at a low temperature and preparing plasmid DNA from these cultures. Alternatively, dark blue colonies are pooled and plasmid DNA is prepared without culturing in order to maintain the integrity of active clones.

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(v) the individual plasmid isolates (or pooled plasmid DNA) can then be introduced into the DNA damage indicator strain which is pre-modified with the same pattern of methylation as in step (ii). The emergence of white colonies on X-gal media at 37°C indicates that the endonuclease variant 5 may be exhibiting cleavage preference towards the site(s) protected by methylation.

The methods described herein are effective in altering 10 cleavage specificity of thermophilic restriction enzymes as these enzymes are less active at 30°C-37°C and are better tolerated by DNA damage indicator cells not protected by methylation. Yet, endonucleases from mesophilic organisms may be 15 engineered by this method by first attenuating the endonuclease activity and/or expression level as described in Examples 2 and 3 of the present invention.

We provide examples of a stringent selection method where 20 an estimated  $10^7$  variants can be rapidly screened in one round. In Example 1, this was applied to increase the substrate specificity of *Bst*YI (5'-RGATCY-3') to single site recognition (5'-AGATCT-3'). After one round (three selection steps), forty-five clones were analyzed *in vitro* for activity and specificity. Of 25 those forty-five clones, two variants were found to exhibit a preference for cleavage of 5'-AGATCT-3' over other *Bst*YI sites. By combining single mutations present in each of these clones, a superior clone designated NN1 was isolated. NN1 displays a 7-fold preference for cleavage of 5'-AGATCT-3' relative to 5'-AGATCC-3' or 5'-GGATCT-3' and cleavage of the 5'-GGATCC-3'

site is not detected (Figure 4). Embodiments of the inventive method are provided below and comprise the following steps, although as the skilled artisan will appreciate, modifications to these steps may be made without adversely affecting the outcome:

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Although not essential, the skilled artisan may choose to consult any available gene homology or protein structural information that pertains to the endonuclease under study or to the appropriate family of endonucleases. This information may be useful in determining and designing the method of mutagenesis.

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1) A given endonuclease gene is mutagenized by methods generally known in the art including any of:

(a) Error-prone PCR (Leung, et al., *Technique* 1:11-15 (1989), Cadwell and Joyce, *PCR Methods Applic.*, 2:28-33 (1992)).

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(b) Hydroxylamine, sodium bisulfite or any other chemical mutagen treatment. (Sambrook, J., et al., *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> ed. (1989)).

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(c) Oligonucleotide directed mutagenesis, preferably the overlap extension PCR mutagenesis method (Morrison and Desrosiers, *Biotechniques* 14:454-457 (1993)).

5                             (d) Assembly PCR. The term "assembly PCR" refers to a process that involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of different PCR reactions occur in parallel in the same vial, with the products of one reaction priming the products of another reaction.

10                             (e) Sexual PCR mutagenesis. The term "sexual PCR mutagenesis" (also known as "DNA shuffling") refers to forced homologous recombination between DNA molecules of different but highly related DNA sequence *in vitro*, caused by random fragmentation of the DNA molecule based on sequence homology, followed by fixation of the crossover by primer extension in a PCR reaction. (Stemmer, *Proc., Natl. Acad. Sci., USA* 91:10747-10751 (1994)).

15                             (f) *In vivo* mutagenesis. The term "*in vivo* mutagenesis" refers to a process of generating random mutations in any cloned DNA of interest which involves the propagation of the DNA in a strain of *E. coli* that carries mutations in one or more of the DNA repair pathways. These mutator strains have a higher random mutation rate than that of a wild-type strain. Propagating the DNA in one of these strains will generate random mutations within the DNA. (Long-McGie, et al., *Biotechnol. Bioeng.* 68:121-125 (2000)).

20                             (g) Cassette mutagenesis. The term "cassette mutagenesis" refers to any process for replacing a small region of a double-stranded DNA molecule with a synthetic

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oligonucleotide cassette that differs from the native sequence. The oligonucleotide often contains completely or partially randomized native sequence. (Dorner, et al., *J. Mol. Biol.* 285:1515-1523 (1999)).

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(h) Recursive ensemble mutagenesis. The term "recursive ensemble mutagenesis" refers to an algorithm for protein engineering (protein mutagenesis) developed to produce diverse populations of phenotypically related mutants whose members differ in amino acid sequence. This method uses a feedback mechanism to control successive rounds of combinatorial cassette mutagenesis (Arkin and Youvan, *Proc. Natl. Acad. Sci., USA* 89:7811-7815 (1992)).

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(i) Exponential ensemble mutagenesis. The term "exponential ensemble mutagenesis" refers to a process for generating combinatorial libraries with a high percentage of unique and functional mutants, wherein small groups of residues are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins (Delegrave and Youvan, *Biotechnology Res.* 11:1548-1552 (1993)) and random and site-directed mutagenesis (Arnold, *Curr. Opin. Biotechnol.* 4:450-455 (1993)).

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Each of these techniques is described in detail in the cited references herein incorporated by reference.

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2) The mutagenized endonuclease gene library is prepared and cloned into an expression vector by standard

techniques. The expression vector should be capable of inducible over-expression and may exhibit low-level constitutive expression of the endonuclease library. One such vector is pAGR3 (New England Biolabs, see Figure 6) containing a Ptac promoter, the lacI<sup>q</sup> gene, a four-fold repeat of the rrnb transcription terminator upstream of the promoter, a Co/E1 origin of replication and an ampicillin resistance gene. Other suitable expression vectors may contain an IPTG-inducible promoter (P<sub>lac</sub>, Ptac, Ptrc), or a T7 promoter. Examples of suitable T7 vectors include pET21a, pET21at (a pET21a derivative constructed at New England Biolabs where 4 copies of the rrnb transcription terminator are inserted upstream of the T7 promoter) and pAI17 (New England Biolabs, see Figure 7). When such T7 vectors are employed, protein overexpression occurs in a bacterial strain carrying the T7 RNA polymerase gene normally regulated by lacI. (e.g. ER2744 [fhuA2 lacZ::T7 gene1 glnV44 e14- rfbD1? relA1? endA1 spoT1? thi-1 Δ(mcrC-mrr)114::IS10] or ER2848 [F' proA+B+ lacI<sup>q</sup> Δ(lacZ)M15 zzf::Tn10(TetR) fhuA2 lacZ::T7 gene1 glnV44 e14- rfbD1? relA1? endA1 spoT1? thi-1 Δ(mcrC-mrr)114::IS10] New England Biolabs). However, other inducible expression systems and prokaryotic hosts may be substituted.

3) The mutagenized endonuclease library may be introduced into a bacterial strain pre-modified with a non-cognate pattern of DNA methylation. Preferably, pre-modification consists of transforming a bacterial strain with one or more plasmids carrying a methylase gene and subsequently preparing those cells for transformation of the endonuclease

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library. For example, ER2502 ([*fhuA2 ara-14 leu Δ(gpt-proA)62 lacY1 glnV44 galK2 rpsL20 endA1 R(zgb210::Tn10)Tet S xyl-5 mtl-1 Δ(mcrC-mrr)HB101*] New England Biolabs) may be used when using a vector with an IPTG-inducible promoter; although, any strain may be used so long as it is tolerant of the imposed DNA methylation state and it is capable of efficient chemical transformation and electroporation. Other suitable strains include, but are not limited to, JM107, HB101, NM522, ER1821, ER2267, ER2744 and ER2848. When employing a vector with a T7 promoter, ER2744 and ER2848 are preferred due to the presence of an IPTG-inducible T7 RNA polymerase gene. Upon endonuclease library introduction, surviving strains are selected on Luria-Bertani (LB) agar plates containing the inducer molecule, an antibiotic to select the endonuclease plasmid and an antibiotic to ensure maintenance of the methylase plasmid(s).

4) Surviving colonies are pooled and plasmid DNA is prepared from the cells. The methylase plasmids are destroyed by digestion with at least two restriction enzymes. The pooled endonuclease library DNA is introduced into a DNA damage indicator strain, for example, ER1992 (NEB#907), which contains the *dinD1:lacZ* gene fusion (U.S. Patent No. 5,498,535). Other DNA damage indicator strains may be substituted for the above where the reporter gene is fused to a promoter induced by the SOS repair response. Other DNA damage-inducing promoters which can be used include *dinA* (Iwasaki, et al., *J. Bacteriol.* 172:6268-6273 (1990)) and *dinG* (Lewis, *J. Bacteriol.* 174:5110-5116 (1992)). Other

indicator/reporter genes which can be fused to any of the above promoters include alkaline phosphatase (*phoA*) (Hoffman and Wright, *Proc. Natl. Acad. Sci. USA*, 82:5107-5111 (1985)), luciferase (*lux*) (Engelrecht, *Science* 227:1345-1347 (1985)),  $\beta$ -glucuronidase (Metcalfe, *Gene*, 129:17-25 (1993)), aminoglycoside phosphotransferase (Ward, et al., *Mol. Gen. Genet.*, 203:468-478 (1986)), and endoglucanase (Bingle, et al., *Can. J. Microbiol.*, 39:70-80 (1993)). Where the *lac Z* reporter gene system is utilized, transformants are selected at a predetermined temperature (for example 30°C-42°C) on agar plates containing an antibiotic to select for the endonuclease plasmid and X-gal to serve as the substrate for blue color development upon induction of the SOS response and expression of the *dinD1::lacZ* gene fusion. Blue cells are presumed to be carrying active endonuclease variants that survived the first selection in the presence of the non-cognate pattern of DNA methylation.

5) Cells determined to be carrying the active restriction endonuclease gene are selected and cultured in order to isolate a minimal amount of plasmid DNA for a subsequent transformation. Alternatively, indicator colonies that are positive for active endonuclease are pooled and plasmid DNA is prepared without culturing in order to maintain the integrity of active clones.

6) The endonuclease clones derived from indicator colonies that are positive are transformed into the DNA damage indicator strain now carrying the same methylase plasmid(s) as

in step 3 to provide the same pattern of methylation protection as in the first genetic selection. For example, transformed cells are plated on LB-agar containing the indicator substrate, an antibiotic to select the endonuclease plasmid and an antibiotic to maintain the methylase plasmid(s). If the non-cognate pattern of methylation protects the cellular DNA from variant endonuclease cleavage, then the indicator response for colonies that are positive for the active endonuclease will not be induced and colonies that are negative will result. For example, the emergence of white colonies of a *dinD1::lacZ* strain suggests that the endonuclease variant displays cleavage preference towards the recognition sequence(s) protected by the non-cognate methylase(s).

7) To further analyze the novel endonuclease, aliquots of individual colonies may be taken for plasmid DNA isolation and sequencing. In one embodiment, the aliquots are taken from white colonies cultured at 30°-37°C for 6-16 hours. The remaining cells may be lysed and the resulting extract analyzed for endonuclease activity and specificity towards an appropriate DNA substrate. The DNA substrate should allow distinction between restriction of the original recognition sequence(s) and a preference for the desired recognition sequence(s).

8) Endonuclease variants displaying a preference for the desired recognition sequence(s) may be sequenced to identify those genetic changes and amino acid substitutions responsible for alteration of the enzyme specificity/recognition site preference.

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9) Those skilled in the art of protein engineering will be able to evaluate the importance of each of the genetic changes present in the selected clones and use this information to 5 rationally design improved endonuclease variants with desired enzymatic properties. The mutations responsible for alteration of enzyme specificity are identified by inference and/or site-directed mutagenesis. The mutations deemed important for altering substrate specificity are combined into one 10 endonuclease clone with the objective of creating a superior endonuclease variant. This variant or variants selected directly from one round can be mutagenized further and subjected to a subsequent round of selection(s) until the desired variant is isolated. At any point in the process, the skilled artisan may 15 choose to make site-directed changes to the endonuclease gene in order to maximize the efficiency of the selection process.

The references referred to above and below are hereby incorporated by reference herein.

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The following Examples are provided to aid in the understanding of embodiments of the invention and are not intended as a limitation thereof.

**Example 1****Alteration of *Bst*YI performance to 5'-AGATCT-3' specificity by random mutagenesis, genetic selection and site-directed mutagenesis.**

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The endonuclease *Bst*YI recognizes and cleaves all hexanucleotide sequences described by 5'-RGATCY-3' with similar proficiency. In this Example, the genetic selection method of the present invention was applied to isolate one or more *Bst*YI variants with cleavage preference towards AGATCT. The sequence AGATCT (Figure 1B) was methylated at the N4 position of cytosine by the *Bg*/II N4-cytosine methyltransferase (Brooks and Roberts, *Nucleic Acids Res.* 10:913-934 (1982), Erlich, et al., *J. Bac.* 169:939-943 (1987)). Methylation of the N4 position of cytosine is known to inhibit cleavage of the AGATCT DNA sequence by *Bst*YI. The *Bg*/II methylase gene (*bg*/IIM) from *Bacillus globigii* (ATCC 49670) was isolated from pUC-BglIIM (Anton, et al., *Gene* 187:19-27 (1997)) by *Bam*HI digestion and subsequently filled-in with Klenow fragment to produce blunt ends. The blunt-ended *bg*/IIM fragment was ligated into *Sma*I-digested/CIP-treated pSYX20 (Morgan, et al., *Gene* 183:215-218 (1996)). This clone, pSYX-BglIIM, was employed during the genetic selection procedure to provide methylation protection where indicated.

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The *Bst*YI endonuclease gene (*bst*YIR) was amplified from pET21at-*Bst*YIR (U.S. Patent No. 6,403,354 B1) by error-prone PCR (Leung, et al., *Technique* 1:11-15 (1989), Cadwell and Joyce, *PCR Methods Applic.* 2:28-33 (1992)). Two sets of PCR

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conditions were selected to give two levels of mutagenesis of the *bstYIR* gene (Shafikhani, et al., *Biotechniques* 23:304-310 (1997)) resulting in mutagenic libraries A1 and A2.

5           A1 PCR conditions were as follows: 8 ng plasmid template per 100 µl reaction, 0.4 µM forward and reverse primers, 5U *Taq* DNA polymerase, 7 mM MgSO<sub>4</sub>, 0.15 mM MnCl<sub>2</sub>, 0.2 mM dATP, 0.2 mM dGTP, 1.0 mM dCTP, 1.0 mM dTTP, 10 mM KCl, 10 mM (NH<sub>4</sub>)SO<sub>4</sub>, 20 mM Tris-HCl (pH 8.8 @ 25°C) and 0.1% Triton X-100. Thermocycling parameters were: 1 cycle for 3 min @ 94°; 10 15 cycles (1 min @ 94°C, 1 min @ 50°C, 1 min @ 72°C) and 1 cycle for 7 min @ 72°C. A Perkin-Elmer 2400 thermocycler was used for all PCR reactions. A2 PCR conditions were exactly as A1 conditions except 30 cycles of amplification were used.

15          Sequencing the 612 bp *bstYIR* gene of genetically selected clones revealed an average of 5 nucleotide substitutions within the A1 mutagenic library and 11 nucleotide substitutions within the A2 mutagenic library. No frameshift mutations were observed in any of the sequenced clones. However, the majority 20 of clones contained a stop codon, presumably allowing the *BstYI* variants to be tolerated in the presence of a non-cognate pattern of methylation. The amber stop codon TAG was found disproportionately, explaining why endonuclease activity was detected in each of the clones as this stop codon is suppressed 25 to a significant degree due to the *glnV44* locus present in both selection strains ER2502 (NEB#1149; New England Biolabs, Inc., Beverly, MA) and ER1992 (NEB#907; New England Biolabs, Inc., Beverly, MA). The *glnV44* locus is responsible for glutamine incorporation at UAG via expression of a suppressor

tRNA<sup>Gln</sup> molecule. The mutagenized *bstYIR* gene libraries A1 and A2 were digested with *Xba*I/*Xho*I and each were ligated into 50 ng *Xba*I/*Sa*I-digested, CIP-treated pAGR3 to create expression vector libraries pAGR3BstYIR-A1 and pAGR3BstYIR-A2. Ligation was carried out for 16 hours at 16°C followed by heat-denaturing the ligase for 30 min at 65°C. The 20 µl ligation reactions were drop-dialyzed against de-ionized water for 4 hours. Then 10% of each reaction was mixed with 40 µL ER2502 [pSYX-BglIIIM] cells prepared for electroporation (BioRad Gene Pulser II protocol). The cell/DNA mix was electroporated at 1.8 kV in a 0.1 cm cuvette (BioRad) followed by addition of 1.0 ml SOC media. The cell suspension was incubated at 37°C for 1 hour and plated (160 µl x 6 plates) on LB-agar containing 100 µg/ml ampicillin, 15 µg/ml tetracycline and 0.3 mM IPTG. The plates were incubated 16 hours at 37°C and survivors on each plate were pooled by LB broth resuspension and plasmid DNA was prepared from the cells on each plate. The plasmid DNA from each plate was digested with *Bam*HI, *Spe*I and *Sa*I to eliminate pSYX-BglIIIM from each library. After heat denaturation of the restriction enzymes (20 min, 80°C), an aliquot of each digestion mix was transformed into 20 µl ER1992, the DNA damage indicator strain. Each transformation mix was plated on 4 LB-agar plates containing 100 µg/ml ampicillin and 40 µg/ml X-gal. Clones from the A1 expression library produced 4-8 dark blue colonies per plate while clones from the A2 expression library produced 1-4 dark blue colonies per plate. These individual dark blue colonies were cultured at 30°C for 3 hours in LB and Amp and plasmid DNA was prepared from each culture. The individual DNA preparations were

transformed into ER1992 chemically-competent cells carrying pSYX-BglIIM. The transformations were plated on LB-agar containing Amp, Tet and X-gal and incubated for 16 hours at 37°C. Individual white colonies were cultured at 37°C, induced with 0.5 mM IPTG for 3 hours and assayed for endonuclease activity and preference for cleavage of AGATCT. The activity/specifity assay was conducted as follows:

Ten milliliters of induced culture were pelleted and the supernatant was removed. The cell pellet was resuspended in 1.0 ml sonication buffer: 10 mM Tris-HCl (pH 7.5 @ 25°C), 10 mM  $\beta$ -mercaptoethanol, 0.1 mM EDTA. The cell suspension was sonicated twice for 25 sec using an Ultrasonics, Inc. Cell Disruptor. The cell debris was pelleted for 10 min at 14,000 rpm. Two microliters of the resulting extract was added to the following 25  $\mu$ l DNA cleavage reaction: 0.5  $\mu$ g pUCAdenoXba substrate, 1x NEB *Bst*YI buffer (10 mM Tris-HCl (pH 7.9 @ 25°C), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol), 0.1 mg/ml BSA. The substrate pUCAdenoXba (New England Biolabs, Inc., Beverly, MA) consists of an Adenovirus-2 DNA fragment from *Xba*I site 10,579 to *Xba*I site 30,455 cloned into the *Xba*I site of pUC19. The circular substrate is 22,562 bp and contains 18 *Bst*YI sites, 4 *Bgl*II sites and 4 *Bam*HI sites. As demonstrated in Figure 3 (lanes 4 and 9), the digestion patterns of *Bst*YI and *Bgl*II are distinctly different. Of 45 selected clones, 2 *Bst*YI variants displayed a digestion pattern indicating a preference for cleavage of AGATCT. The amino acid substitutions of clone 9 were R2opal/K49R/K87R/K133N. (R2opal = Arginine codon at position 2 changed to the TGA stop codon. Tryptophan is

inserted at UGA at a low frequency by the tryptophan-specific tRNA.) The amino acid substitutions of clone A1c were Q28H/S172amber/Y176C. (S172amber = Serine codon at position 172 changed to the TAG stop codon). Assuming that glutamine was being incorporated by amber suppression, the codon at position 172 of clone A1c was changed to CAG and the clone was renamed clone 121. Site-directed mutagenesis of the wild-type *bstYIR* gene determined that the substitutions of K133N and S172Q or S172N are responsible for alteration of *BstYI* specificity. The two substitutions, K133N and S172N, were combined in one *BstYI* variant that was designated NN1.

NN1 was purified from strain ER2744 [pET21at-NN1, pACYC-BstYIM, pCEF8] in the following manner: 500 ml cells induced with 0.5 mM IPTG for 3 hours at 37°C were pelleted and the supernatant was removed. The cell pellet was resuspended in 10 ml sonication buffer: 10 mM Tris-HCl (pH 7.5 @ 25°C), 10 mM β-mercaptoethanol, 0.1 mM EDTA. The cell suspension was sonicated 6 times for 25 sec. After sonication, 25 mM NaCl was added and the cell extract was heated at 65°C for 30 min to denature *E.coli* proteins. The heated extract was centrifuged at 15,000 rpm for 30 minutes and the clarified supernatant was retained. Glycerol was added to a final concentration of 5% before loading the supernatant onto a Heparin-Sepharose FF column equilibrated with 10 mM Tris-HCl (pH 7.5 @ 25°C), 25 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mM EDTA and 5% glycerol. Fractions were eluted with a 0.025-0.8 M NaCl gradient. Fractions containing >90% endonuclease as determined by SDS-PAGE analysis were pooled and the NaCl

concentration was adjusted to 0.3 M. The pooled fractions were allowed to flow through a DEAE-Sepharose column to accomplish DNA/RNA removal. The DEAE-Sepharose column was pre-equilibrated with 10 mM Tris-HCl (pH 7.5 @ 25°C), 0.3 M  
5 NaCl, 10 mM  $\beta$ -mercaptoethanol, 0.1 mM EDTA and 5% glycerol. The DEAE flow-through was dialyzed overnight at 4°C in 20 mM Tris-HCl (pH 7.5 @ 25°C), 100 mM KCl, 10 mM  $\beta$ -mercaptoethanol, 0.1 mM EDTA and 5% glycerol. Glycerol was added to a final concentration of 50% and the purified protein  
10 was stored at -20°C. NN1 displays an even greater preference for cleavage of AGATCT than clones 9 or 121 and purified NN1 protein maintains the same thermostability as wild-type *Bst*YI.

15 By combining the genetic selection method of the present invention, interpretation of the selected genetic alterations by a skilled artisan and site-directed mutagenesis, a *Bst*YI variant was isolated that preferentially recognizes and cleaves 5'-AGATCT-3' (Figures 3 and 4). NN1 displays a 7-fold preference for recognition and cleavage of 5'-AGATCT-3' relative to 5'-AGATCC-3' or 5'-GGATCT-3' and recognition and cleavage of the  
20 5'-GGATCC-3' (SEQ ID NO:6) site is not detected. (Relative amounts of cleavage were calculated by quantifying the rate of product formation displayed in Figure 4).

**Example 2****Selection of NotI variants with altered specificity for DNA recognition and cleavage**

5

The recognition sequence of NotI is 5'-GCGGCCGC-3'.

10 Restriction endonucleases with 8-bp recognition sites are rarely found in nature. Therefore, development of such enzymes by protein engineering is of considerable commercial interest. The method of the present invention is especially suited for this type of endeavor since an extensive range of DNA methylases are available that will modify derivations of the wild-type NotI 15 recognition sequence. However, randomly choosing a non-cognate methylase to employ in the genetic selection of NotI variants imposes an unfair bias on the evolution process.

20 Instead, a preliminary study was conducted to isolate a NotI variant with relaxed specificity. This work was modeled after a study of EcoRI substrate specificity where important amino acid residues were identified using a DNA-damage indicator strain expressing the cognate EcoRI methylase (Heitman and Model, 25 *EMBO J.* 9:3369-3378 (1990)). In the case of the NotI study, the EagI methylase served as the "cognate" methylase since it modifies the sequence 5'-NCGGCCGN-3' and protects the host genomic DNA from cleavage by wild-type NotI.

25

**I. Isolation of NotI variant 44-2A**

30 The EagI methylase clone (pACYC184EagIM) was isolated from the NotI overproduction strain (NEB #816 and U.S. patent No. 5,371,006) and transformed into DNA-damage indicator

strain ER1992. The wild-type *notIR* gene was subjected to error-prone PCR mutagenesis with the following conditions: 10 ng plasmid template pAII17-NotIR, 0.4  $\mu$ M T7 promoter and T7 terminator primers, 5U *Taq* DNA polymerase, 7 mM MgSO<sub>4</sub>, 0.2 mM dATP, 0.2 mM dGTP, 1.0 mM dCTP, 1.0 mM dTTP, 10 mM KCl, 10 mM (NH<sub>4</sub>)SO<sub>4</sub>, 20 mM Tris-HCl (pH 8.8 @ 25°C) and 0.1% Triton X-100. Thermocycling parameters were 1 cycle for 3 min @ 94°C, 25 cycles (1 min @ 94°C, 1 min @ 50°C, 1 min @ 72°C) and 1 cycle for 7 min @ 72°C. The PCR product was digested with *Xba*I and *Sa*I, gel-purified and cloned into pAGR3. The pAGR3 mutagenic library was transformed into ER1992 [pACYC184EagIM] cells by electroporation and plated on LB-agar containing 100  $\mu$ g/ $\mu$ L ampicillin, 33  $\mu$ g/ $\mu$ L chloramphenicol and 80  $\mu$ g/ $\mu$ L X-gal. Of approximately 60,000 transformants, 10 blue colonies were isolated. The endonuclease clones present within these colonies were amplified by colony PCR and cloned into T7 expression vector pAII17. The substrate specificity of each clone was analyzed by overexpression within ER2744 carrying pACYC188-EagIM and pSYX20-HhaIM. The cellular extract of clone 44-2A produced an alternate cleavage pattern when incubated with substrate pUCAdenoXba (previously linearized by *Pme*I). Specifically, the 16,748 bp fragment produced by wild-type *Not*I was partially digested into 7 kb and 9 kb fragments (see Figure 5). Inspection of the substrate sequence determined that cleavage at 5'-GCAGCTGC-3' and/or 5'-GCTGCAGC-3' may be responsible for production of the alternate restriction fragments.

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DNA sequencing of clone 44-2A revealed nucleotide substitutions that result in the amino acid substitutions P9S, E156K and I201T. We deduced that the relaxed specificity was at least partially caused by the E156K substitution, as this 5 variant could not be isolated in host ER2744 [pACYC-EagIM, pSYX20-HhaIM] (due to cell toxicity). Subsequently, the E156K substitution was isolated in an allele that contained an amber codon at position 37 as a means of attenuation. The Amb37/E156K allele was expressed from pAGR3 in ER1992 10 [pACYC184-EagIM] and the resulting extract produced the same altered pattern of digestion as the original clone, 44-2A, thus confirming the importance of substitution E156K for relaxation of substrate specificity.

15 The altered substrate specificity of E156K was investigated *in vivo* by expressing the BbvI methylase in addition to the EagI methylase. The BbvI methylase modifies the C5 position of the first cytosine in the sequence 5'-GCAGC-3' and 5'-GCTGC-3'. Therefore, modification of 5'-GCAGCTGC-3' and 5'-GCTGCAGC- 20 3' by BbvI methylase is predicted to protect DNA from cleavage by a variant NotI (REBASE: <http://rebase.neb.com>). In fact, modification of the genomic DNA by the BbvI methylase increased the tolerance of host ER2744 to Amb37/E156K expressed from pAGR3. When plated on LB-agar containing 25 increasing levels of IPTG, loss of cell viability was detected at 80 µM IPTG in the presence of BbvI and EagI methylation; whereas, viability was lost at 25 µM IPTG when the host was protected by EagI methylation only. This rapid *in vivo* test serves to give an indication of the altered specificity and aids in

the choice of the appropriate methylase to be employed in the genetic selection of the present invention.

5           **II.   Genetic Selection of NotI Variants Possessing  
5'-GCWGCWGC-3' Specificity**

The "relaxed" NotI variant Amb37/E156K can be subjected to one or more rounds of genetic selection in order to isolate an endonuclease that preferentially recognizes 5'-GCWGCWGC-3' (where W = A or T).

In round one, a mutagenized Amb37/E156K library is ligated into pAGR3 and the resulting clones transformed into ER2744 protected by only the BbvI methylase. The appropriate amount of IPTG in selection one is determined by testing which level results in an adequate number of "active" survivors as revealed in selection two by the indicator strain ER1992. Active clones from selection two are transformed into ER1992 expressing the BbvI methylase. White or light-blue colonies in step three are cultured, cell extract is prepared and the endonuclease activity is analyzed *in vitro* by incubation with an appropriate DNA substrate. Clones displaying an even greater preference for 5'-GCWGCWGC-3' as compared to the parent are sequenced and the responsible amino acid alterations are determined by inference and/or site-directed mutagenesis. The most desired variant(s) are subjected to a second round of genetic selection and so forth. In each subsequent round it is important to increase the *in vivo* selection pressure imposed on the mutagenized gene library. In this example, the level of IPTG can be incrementally increased in each round and/or codon 37

can be restored to CAG thus significantly increasing the *in vivo* DNA-damaging effects of those variants possessing activity outside the spectrum of BbvI methylation. Finally, the desired NotI clone will be sequenced to determine the genetic alterations and the protein will be over-expressed, purified to near homogeneity and characterized in detail.

### Example 3

#### **Selection of endonuclease variants with an alternate specificity for DNA cleavage**

The genetic selection method of the present invention can be applied to engineer an existing endonuclease to recognize and cleave an alternate DNA sequence. As outlined in Figure 1A, an element of the genetic selection procedure is methylation protection of the host genomic DNA by one or more non-cognate DNA methyltransferase. The imposed DNA methylation pattern specifically protects the desired, alternate sequence(s) while allowing the cognate DNA sequence(s) to be efficiently cleaved by the wild-type endonuclease. This critical element can be verified by *in vivo* and *in vitro* studies of the non-cognate DNA methyltransferase(s) and the wild-type endonuclease. Regardless of the temperature optimum of the wild-type endonuclease, the activity of the starting mutagenic library can be attenuated to maximize the efficiency of the genetic selection process. Options include creating an amber codon within the open reading frame, creating an opal codon within the open reading frame, changing the start codon to GTG or mutating the RBS sequence to decrease the translational efficiency. In

addition, the use of a T7 expression vector is especially  
advantageous for achieving low-level constitutive expression of  
the endonuclease library in a DNA-damage reporter strain that  
does not carry a T7 RNA polymerase gene. For example, the  
5 mutagenic library for round one can be cloned into the T7  
expression vector pAII17. Mutagenesis of the endonuclease  
gene can be accomplished by error-prone PCR conditions "A1"  
as described in Example 1. After error-prone PCR amplification,  
the mutagenic library (Rgene\*) is digested with the appropriate  
10 restriction enzymes, ligated into pAII17 and subjected to the  
following genetic selections:

SELECTION 1 - The mutagenic library is introduced into  
strain 1 by electroporation or transformation of chemically-  
15 competent cells. Strain 1 (e.g., ER2744 [pACYC-Mgene]) will  
have been pre-modified with the desired, non-cognate pattern  
of methylation. Survivors can be selected at 25°C-42°C on LB-  
agar plates containing a low-level of IPTG (0-100 µM), Amp and  
an antibiotic to ensure maintenance of the methylase  
20 plasmid(s). Surviving colonies from each plate are pooled and  
plasmid DNA is prepared from each pool. The methylase  
plasmid(s) is destroyed by digestion with multiple restriction  
enzymes.

25 SELECTION 2 - The mutagenic endonuclease sub-libraries  
are transformed into DNA-damage indicator strain such as  
ER1992 and plated at 30°C-42°C on LB-agar containing Amp  
and X-gal. Individual active clones displaying a dark blue colony

phenotype are cultured for 2-16 hours at 30°C-42°C and plasmid DNA is prepared from these cultures.

SELECTION 3 - Individual clones can be transformed into ER1992 [pACYC-Mgene] which has been pre-modified with the same pattern of methylation as in strain 1/selection 1. The transformants can be plated at 30°C-42°C on Amp, X-gal and an antibiotic to maintain the methylase plasmid(s). Individual white colonies are cultured, induced with IPTG and cell extract is prepared. Alteration of endonuclease specificity can be analyzed by adding the cell extract to an *in vitro* DNA cleavage reaction containing a substrate which allows distinction between restriction of the original recognition sequence(s) and a preference for the desired recognition sequence(s).

Endonuclease variants displaying the desired specificity (or partial alteration of specificity) are sequenced and the responsible mutations are determined by inference and/or site-directed mutagenesis. The mutations responsible for alteration of specificity can be combined in one endonuclease clone by site-directed mutagenesis. This clone, or any of the variants selected directly from round one, can be chosen for further improvement by a subsequent round of genetic selections.

During selection 1 of round two, the IPTG level will be increased as compared to round one in order to increase the selection pressure. This will increase the likelihood of eliminating those clones with only a partial alteration of substrate specificity. The selection process can end after round two or can proceed for multiple rounds until the desired endonuclease variant is isolated. With each subsequent round, selection 1 will be made

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more stringent, preferably by increasing the level of IPTG present in the agar plates. Finally, the desired endonuclease clone will be sequenced to determine the genetic alterations and the protein will be over-expressed, purified to near homogeneity and characterized in detail.

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